Up-regulation of HIV coreceptor CXCR4 expression in human T lymphocytes is mediated in part by a cAMP-responsive element

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ABSTRACT **The chemokine and HIV receptor CXCR4 has been shown to play a role in chemotaxis and HIV-1 entry into T cells. Dibutyryl cAMP (DcAMP), an analog of cAMP, has been shown to increase CXCR4 cell surface expression and HIV-1 infectivity, but the molecular mechanism(s) responsible is unknown. Here we show that DcAMP treatment of purified human T lymphocytes increased transcription of CXCR4 mRNA as well as cell surface and intracellular CXCR4 protein expression. DcAMP-mediated stimulation of human PBL increased T-trophic HIV-1 (X4) fusion and viral replication as measured by syncytia formation and p24 levels, respectively. To determine the region(s) of the CXCR4 promoter required for cAMP responsiveness, truncations and point mutations of the CXCR4 promoter (nucleotides −1098 to +59) fused to luciferase were constructed and transiently transfected into human PBL. Deletional analysis demonstrated that the −1098 to −93 region of the CXCR4 promoter construct could be eliminated; the residual (−93 to +59) promoter retained cAMP responsiveness. Site-directed mutagenesis of a putative cAMP-responsive element (CRE) in the 5′ UTR (+41 to +49) significantly and specifically attenuated the ability of DcAMP to drive the minimal CXCR4 promoter. Electrophoretic mobility shift assays demonstrated the formation of a complex between the CREB transcription factor and the putative CXCR4 CRE site. Our findings demonstrate a CRE element within the CXCR4 promoter that regulates CXCR4 transcription in response to changes in cAMP signaling. The cAMP-dependent up-regulation of CXCR4 mRNA results in increased CXCR4 intracellular and cell surface protein expression as well as increased HIV infectivity.—Cristillo, A. D., Highbarger, H. C., Dewar, R. L., Dimitrov, D. S., Golding, H., Bierer, B. E. Up-regulation of HIV coreceptor CXCR4 expression in human T lymphocytes is mediated in part by a cAMP-responsive element. *FASEB J.* 16, 354–364 (2002)**

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THE CHEMOKINE RECEPTOR family is comprised of seven transmembrane-spanning receptors that are coupled to and signal through heterotrimeric GTP binding proteins (1). Individual receptors have both distinct and overlapping specificities for their natural chemokine ligands (2). The chemokine superfamily consists of small molecules (8–10 kDa) that function to selectively attract different subsets of leukocytes and are classified by the configuration of their signature cysteine residues near the amino terminus: CC, CXC, CC/CXC, C, and CX_3C (where X represents any amino acid; ref 3). Chemokine/chemokine receptor binding is necessary for primary immune responses and lymphoid tissue homing.

A member of the chemokine receptor family, CXCR4, is expressed on a variety of leukocyte subpopulations including naive T cells, B cells, monocytes, and neutrophils (4). By binding to its natural ligand stromal cell-derived factor 1α (SDF-1α), CXCR4 has been shown to play a role in neutrophil and lymphocyte chemotaxis (5–7). CXCR4 functions as an HIV-1 coreceptor to mediate infection of T cell tropic HIV strains (8–13); CXCR4, CD4, and the HIV gp120 form a multimolecular complex that plays a critical role in the initial stages of HIV fusion with and entry into T lymphocytes (14). The efficiency of early HIV viral entry greatly affects later viral production, revealed after only a few replicative cycles (15).

Whereas second messengers could potentially affect CXCR4 expression, cAMP has been shown to be an important signaling intermediate regulating CXCR4 cell surface expression and HIV-1 infectivity (16). Increases in intracellular cAMP have been shown to activate cAMP-dependent protein kinase A (PKA) in cellular systems; PKA, in turn, phosphorylates various substrates, including the transcription factor cAMP-responsive element binding protein (CREB) (17, 18). Phospho-CREB is then able to bind to the consensus sequence defining the cAMP-responsive element (CRE), which in turn regulates transcriptional activation of target genes. The mechanisms underlying the

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effect of cAMP on CXCR4 expression, however, are not known.

In this report, we show that CXCR4 mRNA levels and transcriptional activation of the CXCR4 promoter are up-regulated by increasing concentrations of cAMP, induced by treatment with dibutyryl cAMP (DcAMP). We demonstrate that intracellular and cell surface CXCR4 protein expression increases in response to a rise in cAMP concentrations. We demonstrate further that DcAMP treatment of purified, human peripheral blood lymphocytes (PBL) incubated with T-tropic (X4) HIV increased viral fusion and replication as measured by syncytia formation and p24 levels, respectively. Deletional analysis of the CXCR4 promoter revealed a putative cAMP-responsive element; site-directed mutation of the putative CRE resulted in attenuation of DcAMP up-regulated promoter activity. Finally, electrophoretic mobility shift assays (EMSA) supported the hypothesis that the transcription factor CREB bound to the CXCR4 CRE site, an event up-regulated by DcAMP treatment of human PBL and eliminated by site-specific mutation. Our results provide a molecular mechanism underlying the cAMP-dependent up-regulation of cell surface CXCR4 expression and HIV infectivity and suggest that inhibition of cAMP activity may limit early stages of HIV infection.

MATERIALS AND METHODS

Cells

PBL were obtained from healthy human donors, isolated by apheresis, followed by reverse flow elutriation and Ficoll-Hypaque centrifugation, and washed with 1× phosphate-buffered saline (PBS). PBL were resuspended in RPMI 1640 (MediaTech, Herndon, VA) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco-BRL, Gaithersburg, MD), 2 mM L-glutamine, 10 mM HEPES pH7.2, 100 U/ml penicillin, 100 μg/ml streptomycin (MediaTech), and 50 μM 2-mercaptoethanol (Bio-Rad, Hercules, CA), termed 10% RPMI, and incubated at 37°C, 5% CO_2 in air. After overnight incubation, cells were either left untreated or stimulated with DcAMP (Sigma, St. Louis, MO), phorbol 12-myristate-13-acetate (PMA; Calbiochem, La Jolla, CA), and ionomycin (Iono; Calbiochem), as indicated. CD4+ and CD8+ T lymphocytes were purified by negative selection using an indirect magnetic labeling system and the MidiMACS™ columns (Miltenyi Biotec) according to the manufacturer's instructions. Non-CD4+ cells were magnetically depleted from PBL using a mixture of CD8, CD11b, CD16, CD19, CD36, and CD56 antibodies. The resulting purified CD4+ T cell population was routinely 96% CD4+ assayed by direct immunofluorescence using an anti-human fluorescein isothiocyanate-conjugated CD4+ antibody (clone RPA-T4; PharMingen, San Diego, CA). Non-CD8+ cells were magnetically depleted from PBL using a mixture of CD4, CD11b, CD16, CD19, CD36, and CD56 antibodies. The resulting purified CD8+ T cell population was routinely 89% CD8+ as assayed by direct immunofluorescence using a phycoerythrin-conjugated antibody (clone SFC121thyd3; Coulter, Fullerton, CA).

RNase protection assay (RPA)

Total RNA was prepared from PBL using Trizol™ (Life Technologies, Gaithersburg, MD) according the manufacturer's recommended protocol, quantitated using OD_{260}, and subsequently used to analyze mRNA expression with the Riboquant RPA system (human hCR6 probe set; PharMingen) according to the manufacturer's instructions. ^{32}P-labeled antisense RNA probes were synthesized from the human cytokine receptor set 6 template by T7 RNA polymerase. The probe (2.1×10^5 cpm/μl for hCR6) was hybridized in solution overnight in excess to target RNA (2 μg total RNA/treatment) in a total reaction volume of 10 μl. Free probe and other single-stranded RNA were digested with RNAses A + T1 per the manufacturer's protocol. The remaining RNase-protected probes were precipitated, dissolved in 5 μl of sample buffer (PharMingen), and resolved on denaturing polyacrylamide gels, followed by autoradiography for 1 day at −70°C. Bands were quantitated by PhosphorImaging analysis (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software and CXCR4 mRNA levels were normalized to L32 mRNA levels.

Cell surface and intracellular staining

Human PBL, stimulated as described, were harvested by centrifugation for 5 min at 500 *g*. For cell surface staining, cells were resuspended in 1× PBS and incubated with the phycoerythrin-conjugated mouse anti-human CXCR4 antibody (12G5; R&D Systems, Minneapolis, MN) or isotype control antibody for 30 min at 4°C in the dark. After 30 min, cells were washed twice with 1× PBS, resuspended in 1% paraformaldehyde (in 1× PBS), and analyzed by FACS using a Coulter cytometer. Intracellular staining was carried out using the Becton Dickinson Fix-Perm intracellular staining kit (San Jose, CA). Human stimulated cells were resuspended in 100 μl 10% cRPMI-1640 to which 2 ml of 1× FACS™ lysing solution was added. Samples were vortexed and incubated for 10 min at room temperature, followed by centrifugation at 500 *g* for 5 min. Cell pellets were resuspended in 500 μl of 1× FACS permeabilizing solution, vortexed, and incubated for 10 min at room temperature in the dark. Samples were washed by adding 1× PBS (0.5% BSA/0.1% NaN_3), followed by centrifugation for 5 min. Cells were resuspended in 1× PBS (0.5% BSA/0.1% NaN_3) and incubated with anti-CXCR4 antibody (12G5; R&D Systems) for 30 min at 4°C in the dark for 30 min, followed by two washes with 1× PBS (0.5% BSA/0.1% NaN_3), resuspension in 1% paraformaldehyde (in 1× PBS), and acquisition of cells using a Coulter cytometer.

Constructs and transient transfections

pGL-CXCR4(−1098 to +59), pGL-CXCR4(−357 to +59), pGL-CXCR4(−283 to +59), pGL-CXCR4(−230 to +59), pGL-CXCR4(−135 to +59), pGL-CXCR4(−93 to +59), and pGL-CXCR4(−42 to +59) were generous gifts from H. Moriuchi (Department of Pediatrics, Nagasaki University School of Medicine, Nagasaki, Japan) and A. Fauci (NIAID, National Institutes of Health, Bethesda MD). Site-directed mutagenesis was carried out on the pGL-CXCR4(−93) construct using the Quick-Change™ protocol (Stratagene, La Jolla, CA). The desired mutations were verified by sequencing (BioServe, Laurel, MD). Constructs were transfected into human PBL by electroporation using the Cell Porator™ (Life Technologies). Firefly luciferase reporter constructs were cotransfected with a reporter vector that contains a cDNA encoding *Renilla* luciferase (pRL-TK) under the control of the herpes simplex virus thymidine kinase promoter (Promega, Madison, WI). pRL-TK was used to control for transfection efficiency. Human PBL (10^7 cells) were transfected with 50 μg of firefly luciferase construct and 1 μg *Renilla* luciferase construct by electro-

poration (320V and 1180 μF, Cell Porator™ Life Technologies). Cells were incubated for 24 h at 37°C, 5% CO_2 and stimulated as described. The dual luciferase assay (Promega) was performed to determine firefly and *Renilla* luciferase activities in cell lysates. Stimulated cell suspensions were transferred to Eppendorf tubes and pelleted by centrifugation at 500 *g* for 5 min. Cell pellets were washed once with 1× PBS, then lysed with 50 μl of 1× Promega passive lysis buffer. Samples were vortexed for 30 s, incubated at RT for 15 min, and pelleted again for 5 min at 20,000 *g*. The luminescence of 100 μl of luciferase assay reagent added to 20 μl of each lysate was recorded using a Lumat LB9507 luminometer (EG&G Berthold, Gaithersburg, MD). Finally, 100 μl of Stop & Glo reagent was added to the sample and a second luminescence reading was recorded (*Renilla* luciferase).

Nuclear extract preparation and EMSA

Unstimulated and stimulated human PBL (10^7 cells) were centrifuged at 20,000 *g* for 5 min at 4°C, washed once with 1× cold PBS, then resuspended in 400 μl of cold buffer (10 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.2 mM PMSF). Cells were allowed to swell on ice for 10 min, vortexed for 10 s, and centrifuged at 20,000 *g* for 10 s. The pellets were resuspended in 40 μl of cold buffer (20 mM HEPES-KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF) and incubated on ice for 20 min. Supernatants were removed after centrifugation at 500 *g* for 2 min at 4°C and stored at −80°C before use in gel shift assays. EMSAs were carried out as outlined by the manufacturer (Geneka Biotechnology Inc., Montreal, QC). The probes used were as follows:

5′-AGGTAGCAAA*GTGACGCCG*AGGGCCTGGGA-3′ (WT),
5′-GGAAGGGTCTCTAACAGAGGGACGCAGGCG-3′ (SH),
5′-AGGTAGCAAA*GCTTCGGTT*AGGGCCTGGGA-3′ (Mut)

HIV p24 assay

After stimulation, cells were centrifuged at 500 *g* for 10 min at 37°C. The supernatant was removed and cells were resuspended in the HIV virus inoculum at a concentration of 2.5×10^6 cells/ml, using 1000 TCID50 of virus stock/10^6 cells. Cells were incubated for 2 h at 37°C, 5% CO_2 with occasional shaking. Cells were centrifuged at 1500 rpm for 10 min at 37°C and washed twice with 1× PBS (containing 2% FCS, 1% penicillin/streptomycin, 0.1% glucose, and 1% HEPES). Cells were then resuspended in RPMI (containing 10% FCS; 1% penicillin/streptomycin, 2% glutamine, and 20 U/ml IL2) at a concentration of 10^6 cells/ml and incubated at 37°C, 5% CO_2. On days 4, 7, and 11 postinfection, p24 levels in the supernatants were measured by enzyme-linked immunoabsorbent assay (p24 EIA, Coulter Corp., Miami, FL). ELISA data are shown; RT-PCR was also performed to confirm results.

HIV fusion/syncytium formation

HIV fusion was assessed by measuring syncytium formation after incubation of stimulated human PBL with TF228.1.16 cells stably transfected with IIIB envelope (X4). TF228.1.16 is a human lymphoid cell line that stably expresses HIV-1 IIIB/BH10 (T-tropic) envelope (a gift from Z. L. Zonak, GlaxoSmithKline, Philadelphia, PA). These cells were mixed with the stimulated PBL groups at a 1:1 ratio (1×10^5 cells/well each) in 96-well plates in triplicate. They were incubated at 37°C, 5% CO_2, and syncytia were scored at 3 and 6 h. Syncytium were identified and counted in the triplicate samples by examining these cells using a light microscope. The mean values and standard deviation of the triplicate readings are reported.

RESULTS

Dibutyryl cAMP increases induction of CXCR4 mRNA in human PBL

To evaluate the effect of modifying cAMP levels on the cellular expression of CXCR4, human PBL were either left untreated or were treated with DcAMP; RPAs were used to assess CXCR4 mRNA levels (**Fig. 1*A***). Within 1 h, DcAMP treatment increased CXCR4 mRNA, peaking 3 h after stimulation and declining gradually over the ensuing 24 h period examined. Up-regulation of CXCR4 mRNA by DcAMP was evident at concentrations of 10 mM (Fig. 1*A*) as at 100 μM (data not shown). To determine whether the CXCR4 mRNA up-regulation was dependent on new mRNA synthesis, purified PBL were pretreated for 1 h with actinomycin D (ActD), a transcriptional inhibitor, before addition of DcAMP. Treatment with ActD completely abrogated cAMP-dependent up-regulation of CXCR4 mRNA (Fig. 1*A*). CXCR4 mRNA levels declined at a rate greater than in control samples if cells were treated with ActD 2 h after stimulation with DcAMP (Fig. 1*A*). Quantitation of CXCR4 mRNA, normalized to L32 controls using ImageQuant software (Fig. 1*B*), demonstrated that the induction of CXCR4 mRNA expression by cAMP-dependent stimulation of PBL was dependent on transcription and synthesis of a new message and not simply on post-transcriptional stabilization of mRNA.

DcAMP increased cell surface and intracellular CXCR4 protein expression

Having shown that increasing intracellular cAMP concentrations increased CXCR4 mRNA, we used direct immunofluorescence to determine CXCR4 protein expression in intact (**Fig. 2*A–C***) and permeabilized (Fig. 2*B*) cells to reflect cell surface and intracellular expression, respectively. Stimulation of human PBL with DcAMP for 24 h increased CXCR4 protein expression on the cell surface vs. unstimulated cells cultured in medium alone (Fig. 2*A*). An increase in the percentage of cells positive for CXCR4 and the CXCR4 fluorescence intensity per cell was observed; the increase seen at 24 h was increased further at 36 h and persisted until 48 h after initiation of stimulation. DcAMP treatment also resulted in an increase in intracellular CXCR4 protein expression at 24 and 40 h (Fig. 2*B*). Pharmacological increases in cAMP levels increased CXCR4 cell surface expression in purified CD4+ (Fig. 2*C*) and CD8+ (data not shown) T lymphocytes. The effect of DcAMP on CXCR4 was not limited to one subpopulation of T cells.

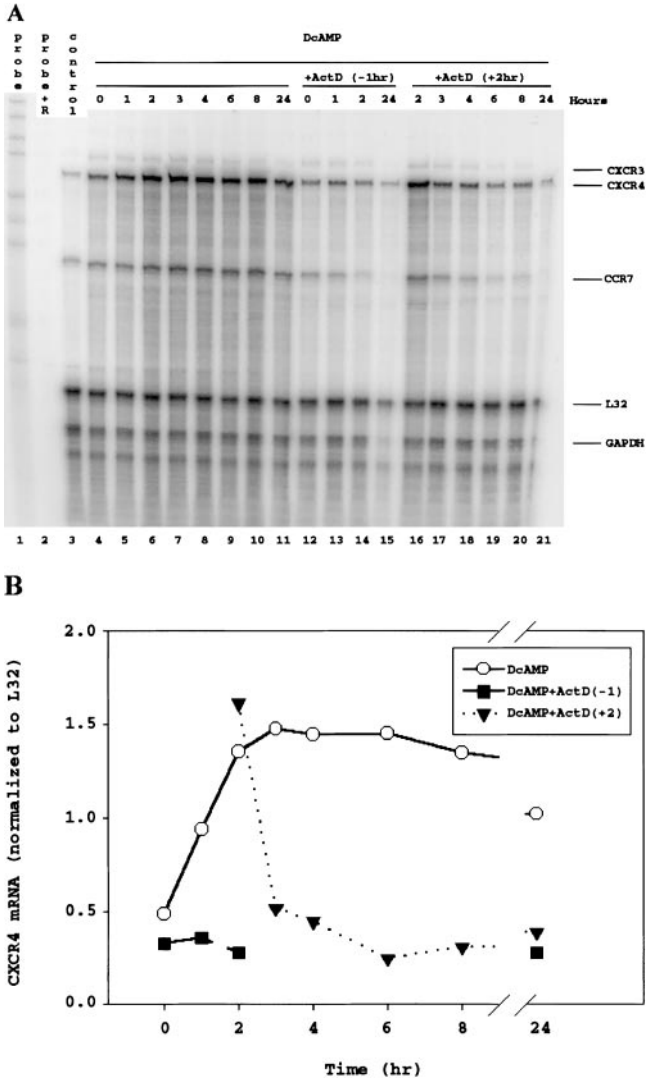


Figure 1. Up-regulation of CXCR4 mRNA by dibutyryl cAMP is actinomycin D sensitive. Human peripheral blood T lymphocytes (PBL) were stimulated with DcAMP (10 mM) for 0, 1, 2, 3, 4, 6, 8, and 24 h (*A*). Actinomycin D (2.5 μg/ml) was added 1 h before (*A*, lanes 12–15) or 2 h after (*A*, lanes 16–21) stimulation. mRNA was prepared from these cells using Trizol™, quantitated using OD_{260}, and analyzed by an RPA (see Materials and Methods). *A)* A representative RPA gel. *B)* CXCR4 mRNA levels normalized to levels of L32 mRNA were quantitated by PhosphorImaging analysis using ImageQuant software. Lane 1: control ^{32}P[UTP]-labeled probe. Lane 2: control ^{32}P[UTP]-labeled probe + RNase. Lane 3: positive control human mRNA (PharMingen). The experiment was repeated three times with similar results.

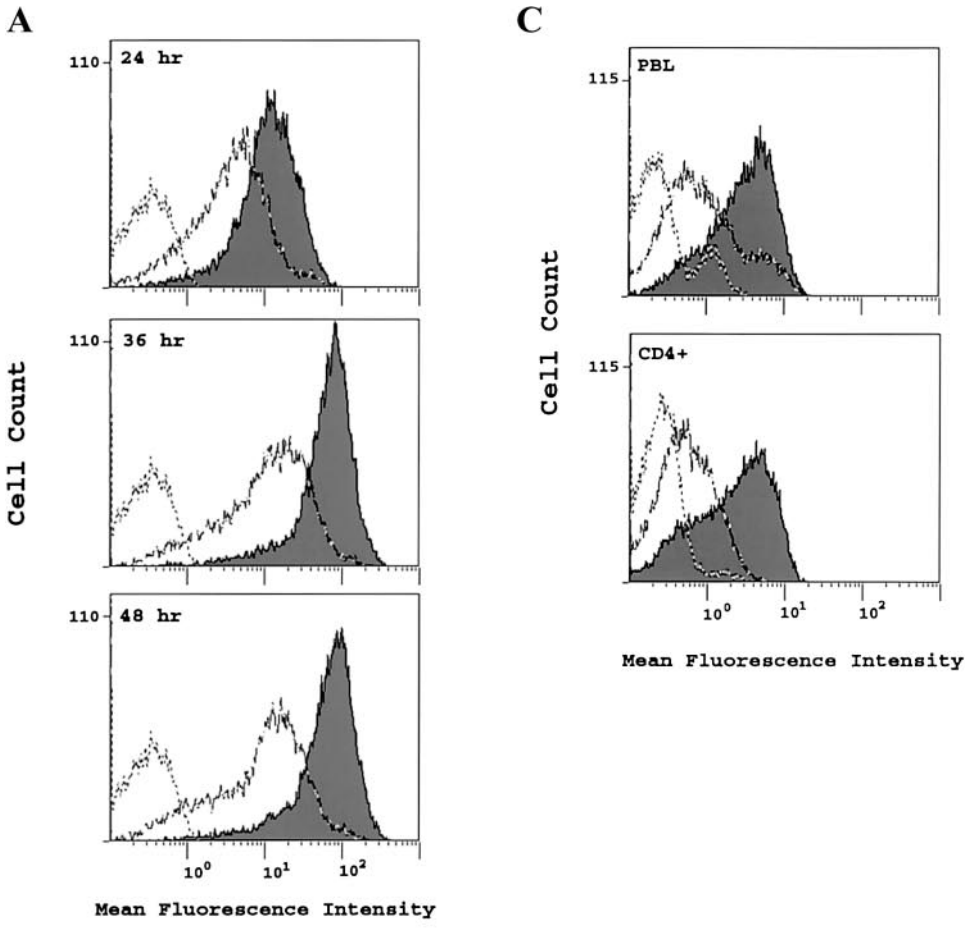
Dibutyryl cAMP increases HIV fusion and replication in human PBL

The effect of increasing cAMP concentration on HIV replication was examined. Human PBL were either left unstimulated or stimulated for 12 h or 24 h with DcAMP or the combination of PMA and ionomycin, agents that activate protein kinase C and increase intracellular calcium concentrations, respectively. The cells were then washed, resuspended in media containing the HIV viral inoculum, and cultured for 2 h (see Materials and Methods). After viral incubation, cells were washed again and cultured at 37°C, 5% CO_2 for 4, 7, and 11 days, after which p24 HIV levels in the culture supernatants were measured by ELISA (**Fig. 3**). Compared with unstimulated samples, cells stimulated for 12 and 24 h with PMA and ionomycin showed no change in p24 levels at 4 days and reduced levels 7 and 11 days after infection. Conversely, stimulation of PBL with DcAMP for either 12 or 24 h resulted in increased p24 levels at 4, 7 (maximal), and 11 days after infection (Fig. 3).

To determine whether the cAMP-dependent increase in HIV replication could be explained by an increase in viral fusion, syncytia formation was quantitated using PBL incubated with TF228 cells stably transfected with and expressing the T-tropic HIV IIIB envelope (X4). Cells were left unstimulated or were stimulated for 24 h with DcAMP, forskolin, PMA, or PMA+ ionomycin. A significant decrease in syncytia formation at 3 (data not shown) and 6 h (**Fig. 4**) was observed in cells stimulated with either PMA or PMA + ionomycin compared with unstimulated controls, consistent with previous findings (19–21); the attenuation in syncytia formation has been attributed to down-modulation of cell surface CXCR4 expression (21). By contrast, we found DcAMP (in a concentration-dependent manner) and the cAMP agonist forskolin stimulation increased syncytia formation at 3 and 6 h (Fig. 4). The cAMP-dependent increase in syncytia formation correlated with increased CXCR4 cell surface expression and with increased HIV replication.

cAMP-dependent regulation of CXCR4 promoter activity in human PBL

To determine the molecular basis of cAMP-dependent regulation of CXCR4 mRNA expression, we analyzed the sequence of the reported CXCR4 promoter. We noted several potential transcription factor binding sites, including a putative CRE in the 5′ UTR upstream of the AUG transcriptional start site (+41 to +49). To identify the *cis*-acting region(s) required to mediate the effects of cAMP, we transiently transfected human purified PBL with a molecular construct containing the CXCR4 promoter sequence (−1098 to +59) or truncations of the CXCR4 promoter fused upstream of the firefly luciferase gene. DcAMP treatment of human PBL transfected with the intact pGL-CXCR4(−1098) plasmid resulted in a fourfold increase in CXCR4 promoter activity vs. unstimulated controls (**Fig. 5**). Truncations of the CXCR4 promoter were tested in similar experiments. Removal of the amino-terminal 1005 nucleotides from the CXCR4 promoter did not significantly affect the responsiveness of PBL to DcAMP: DcAMP treatment of cells transfected with the truncated pGL-CXCR4(−93) retained the ability to drive CXCR4-dependent luciferase activity. Further deletion and expression of the plasmid pGL-CXCR4(−42) failed to permit cAMP-dependent pro-



Treatment	24 h		36 h		48 h	
	%	MFI	%	MFI	%	MFI
EtOH	73.9	8.3	83.5	23.0	79.8	21.6
DcAMP	92.3	16.3	94.1	77.3	93.6	79.5

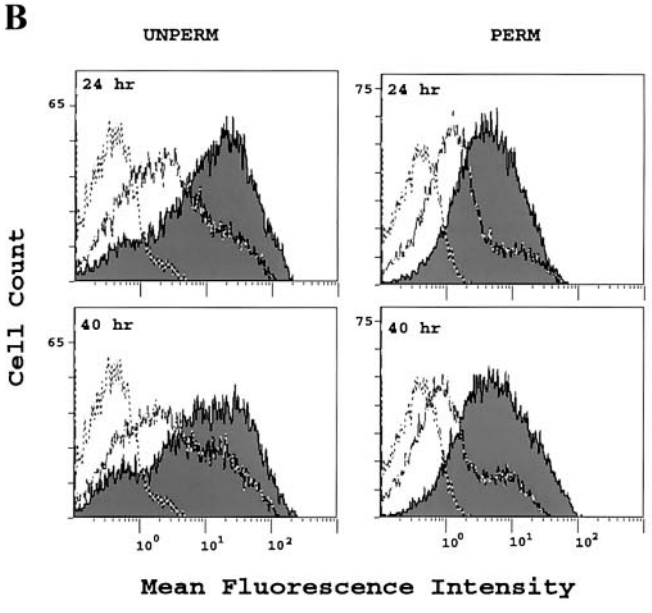


Figure 2. Increased CXCR4 cell surface and total protein levels in DcAMP-stimulated human T lymphocytes. Human PBL were treated in the presence or absence of DcAMP (10 mM) for 24, 36, 40, or 48 h. Cells were labeled with phycoerythrin-conjugated anti-human CXCR4 mAb to quantitate cell surface (*A*) or intracellular (*B*) CXCR4 expression. *A)* Time course of cell surface CXCR4 expression after DcAMP treatment of human PBL. *B)* Intact (UNPERM) or permeabilized (PERM) cells were stained with CXCR4 as outlined (Materials and Methods). *C)* Cell surface immunofluorescence of human PBL and purified CD4+ cells after stimulation with DcAMP for 24 h. In each representative histogram, antibody isotype controls (dotted lines) are compared with CXCR4-specific fluorescence from unstimulated (solid lines/unshaded) and DcAMP-treated (solid line/shaded) samples.

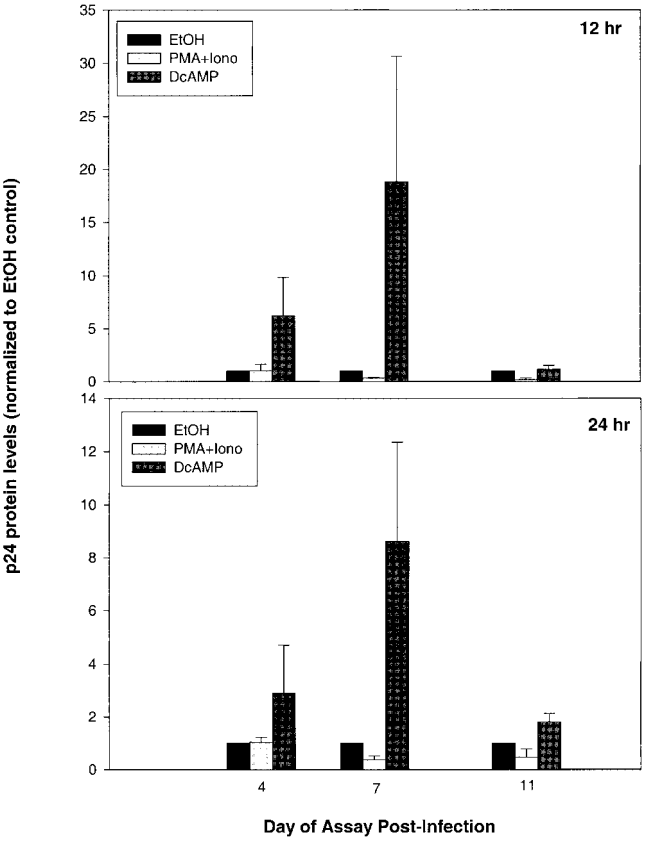


Figure 3. DcAMP-dependent up-regulation of HIV p24 levels in human PBL. After 12 h or 24 h stimulation with either PMA (10 ng/ml) + Iono (1 μM) or DcAMP (10 mM), cells were incubated with HIV viral stock; supernatants were collected on days 4, 7, and 11 after infection to determine p24 viral levels by EIA. Data are shown normalized to unstimulated control samples (EtOH=1.0) and are expressed as the mean average value of 2 (12 h stimulation) or 3 (24 h stimulation) experiments (±SE).

moter activity. Consistent with earlier data (22), PMA and ionomycin treatment of cells transfected with this truncated pGL-CXCR4(−42) promoter construct was unable to induce luciferase activity (Fig. 5).

Identification of a putative CRE site in CXCR4 promoter

To confirm that the putative CRE element noted in the 5′ UTR (+41 to +49) of the CXCR4 promoter was responsible for mediating the DcAMP-dependent up-regulation of CXCR4 promoter activity, site-directed mutagenesis of the putative CRE site within the pGL-CXCR4(−93) construct was performed (**Fig. 6*A***). DcAMP treatment of human PBL transfected with the mutated construct [MutpGL-CXCR4(−93)] demonstrated attenuated luciferase activity compared with cells transfected with the wild-type pGLCXCR4(−93) construct. This attenuated activity was specific for DcAMP in that induction of MutpGL-CXCR4(−93)-driven promoter activity by PMA plus ionomycin was preserved. These results are consistent with a model in which the up-regulation of CXCR4 promoter activity is mediated by cAMP-dependent binding of the transcription factor CREB to CRE, resulting in increased mRNA and protein levels. Our results are consistent therefore with cAMP-dependent transcriptional activation of the CXCR4 promoter, transcription that depends on sequences within the CXCR4 promoter distinct from those responsive to PMA and ionomycin.

CREB forms a complex with CRE in CXCR4 promoter

To determine whether the transcription factor CREB actually binds to the CRE site in the CXCR4 promoter, EMSAs were performed (**Fig. 7**). Nuclear extracts were prepared from human PBL that were unstimulated or stimulated for varying lengths of time with DcAMP. A binding complex was noted in the unstimulated sample (Fig. 7*A*, lane 2; filled arrow) that increased in intensity at 0.5 and 1 h (lanes 3, 4) after DcAMP treatment, then declined by 2 h (lane 5). The DNA–protein complex was supershifted by incubation with the rat anti-human cAMP-responsive element binding protein 1 (CREB-1) antibody (open arrow; compare lanes 6 and 3) and disrupted by unlabeled (lane 7) or shuffled (lanes 8, 9) oligonucleotide probe. No DNA–protein binding complex was observed when a mutant oligonucleotide probe containing point mutations in the putative CRE site was used (Fig. 7*B*, lane 5). A lower band was noted (arrowhead) in every lane that was not disrupted by shuffled oligonucleotide, thus supporting the notion that this band represents a nonspecific interaction. Taken together, our results are consistent with a model in which CXCR4 mRNA induction is regulated by CREB-1 binding to a CRE element in the CXCR4

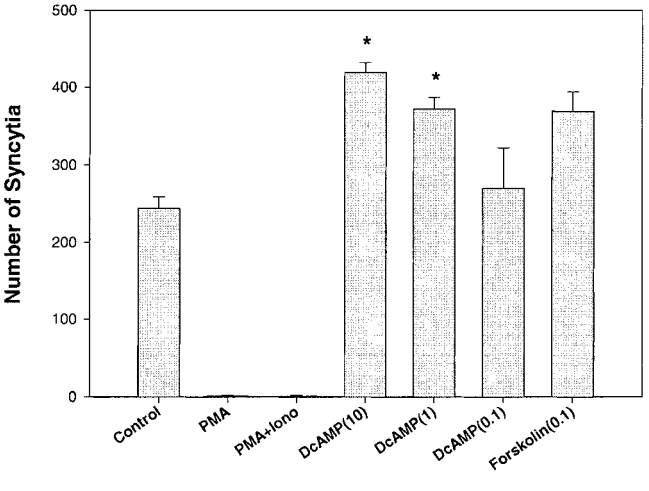


Figure 4. DcAMP and forskolin increases HIV syncytia formation in human PBL. Human PBL were stimulated with DcAMP (10 mM, 1 mM, 0.1 mM), followed by incubation of with TF228 cells stably transfected with the T-tropic HIV IIIB envelope (X4). Envelope-expressing cells were mixed with the stimulated PBL groups at a 1:1 ratio. Syncytia were counted at 6 h and are reported as 3 mean values (±SD) of triplicate determinations. $*P < 0.01$ in $n = 2$ experiments.

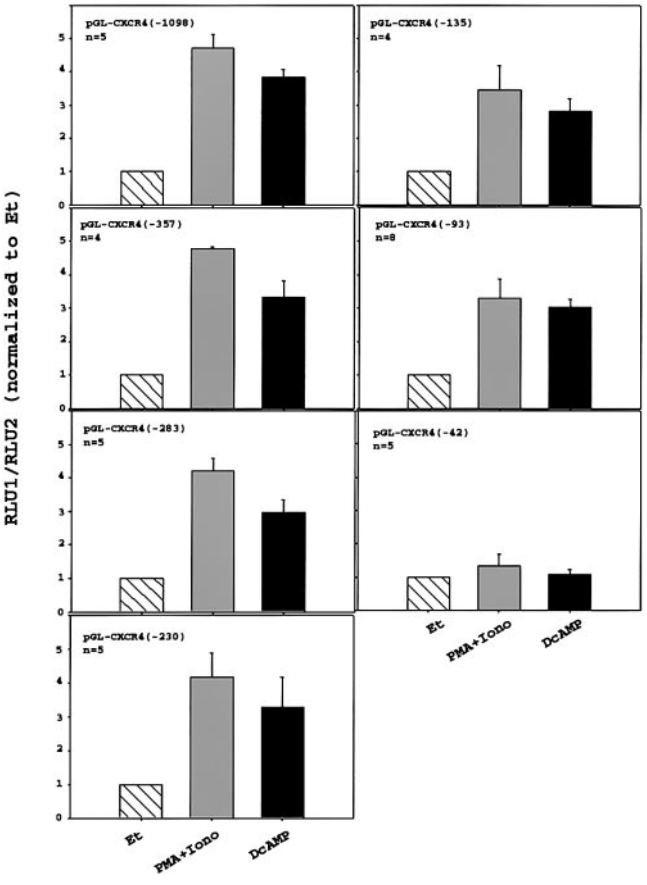


Figure 5. CXCR4 promoter activity in transiently transfected human T lymphocytes. CXCR4 promoter-luciferase constructs were cotransfected with pRL-TK into human PBL (10^7 cells) as described in Materials and Methods. After 24 h incubation, cells (10^6 cells/sample) were incubated in ethanol diluent control (Et, striped bars), PMA (10 ng/ml), and ionomycin (Iono, 1 μM) (gray bars) or DcAMP (10 mM, black bars). 12 h later, cells were harvested and luciferase activity was determined. Firefly luciferase (RLU1) was normalized to *Renilla* luciferase (RLU2 values) to control for transfection efficiency. The mean (±SE) value of *n* experiments normalized to the Et control samples is shown.

promoter, which in turn results in increased intracellular and cell surface protein expression.

DISCUSSION

Chemokines constitute a large family of chemotactic cytokines that act via G-protein-coupled receptors to regulate diverse biological processes including leukocyte trafficking, angiogenesis, hematopoiesis, and organogenesis (23–25). Five major chemokine receptor classes (CC, CXC, CC/CXC, C, and CX_3C) have been classified and 15 chemokine receptor subtypes within these five groups have been identified. Among the CC and CXC families are CCR5 and CXCR4, the principal coreceptors for entry of M-tropic (R5) and T-tropic (X4) HIV-1 virus into macrophages and T lymphocytes, respectively (8, 26, 27). The natural ligand for CXCR4, SDF-1α (CXCL12), was identified and found to be highly expressed in fetal liver and bone marrow stromal cells (5, 25, 28). In contrast to other chemokines that can bind to and signal through individual chemokine receptors, SDF-1α binds to and signals through CXCR4 alone. Unlike other chemokine receptors with overlapping specificity and function, targeted gene disruption of CXCR4 results in mice that die perinatally: the lack of CXCR4 expression results in impairment of cerebellar development and gastric vascularization (29–32). Based on the phenotype of these mice, it was suggested that SDF-1α/CXCR4 plays an essential role in fetal development of brain, heart blood, and stomach. Later studies with mice deficient in CXCR4 expression have demonstrated a requirement of CXCR4 expression for the retention of B lineage and granulocytic precursors within the bone marrow microenvironment (33, 34).

Although these transgenic studies have revealed several developmental processes and signaling pathways to which CXCR4 expression is critical, much remains unknown regarding the regulatory mechanisms governing CXCR4 expression. SDF-1α has been shown to down-modulate cell surface CXCR4 protein levels by activating protein kinase C, thus increasing receptor internalization (21, 35). More recently, cAMP was shown to increase CXCR4 cell surface expression (16) by an unknown mechanism, the nature of which was the focus of this current study. Analyses of the transcriptional regulation of CXCR4 expression revealed that

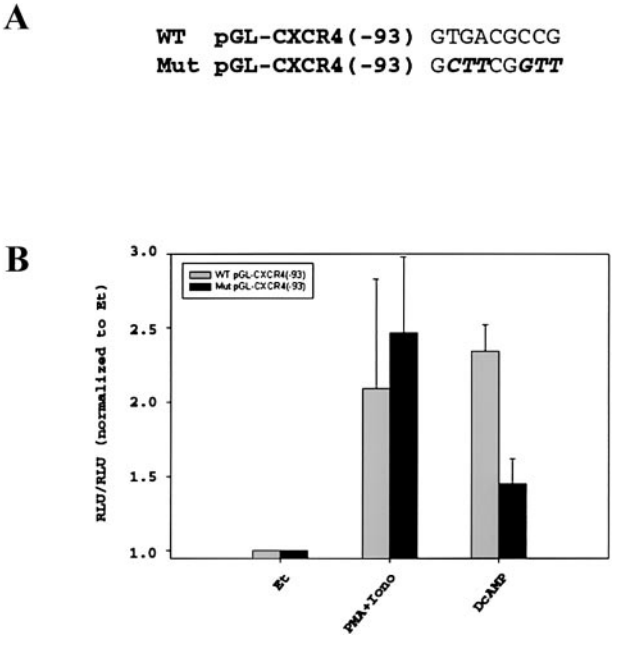


Figure 6. Site-directed mutation of putative CRE element in CXCR4 promoter significantly inhibits DcAMP up-regulated promoter activity in human T lymphocytes. *A*) Alignment of the CXCR4 WT putative CRE sequence and the mutant (Mut) sequence are shown (inset). *B*) The wild-type (WT) pGL-CXCR4(−93) (gray bars) and mutant (Mut) pGLCXCR4 (−93) (black bars) constructs were cotransfected with pRL-TK into human PBL (10^7 cells). Cells were treated and luciferase was analyzed as described for Fig. 5.

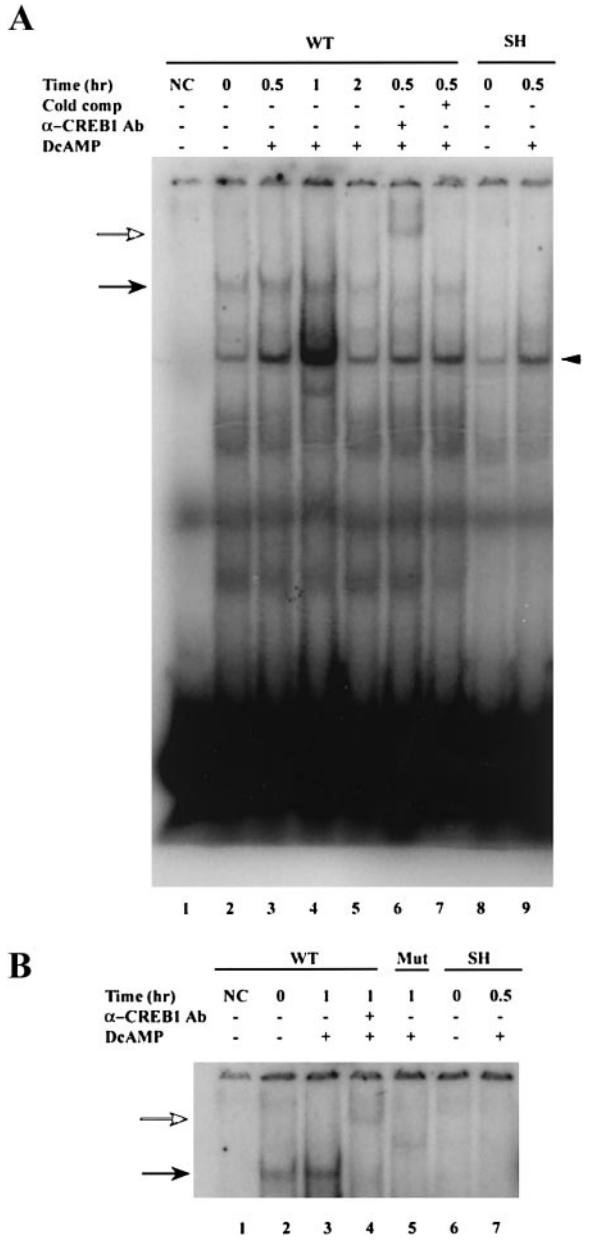


Figure 7. CREB forms a complex with CRE in CXCR4 promoter. Nuclear lysates were prepared from human PBL treated with DcAMP (10 mM) for 0, 0.5, 1, and 2 h (see Materials and Methods). An oligonucleotide probe containing the CXCR4 putative CRE site (WT) was labeled with [γ-^{32}P]ATP, incubated with nuclear lysates, and analyzed by EMSA (*A*, lanes 2–5; *B*, lanes 2–4). The DNA–protein complex (solid arrow) was supershifted by a rat anti-human CREB1 antibody (Geneka Bio/Technology) (open arrow; *A*, lane 6; *B*, lane 4), disrupted by an unlabeled oligonucleotide competitor (*A*, lane 7), and was absent in samples incubated with a shuffled (SH) oligonucleotide probe (*A*, lanes 8, 9; *B*, lanes 6, 7). A nonspecific band (arrowhead) was present in all lanes. An oligonucleotide probe with point mutations in the CXCR4 CRE site (Mut; see Fig. 4) was incubated with prepared nuclear extract and failed to produce the DNA–protein complex (*B*, lane 5). Negative control (NC) samples with no nuclear extract are also shown (*A*, lane 1; *B*, lane 1).

IL-2 regulates CXCR4 mRNA levels (36) and have identified several transcription factors that function as positive (NRF-1, Sp1, USF/c-Myc) and negative (YY1) regulators of CXCR4 gene expression (22, 37, 38). Our findings extend this group of CXCR4 transcriptional regulators to include CREB-1.

We observed basal expression of CXCR4 mRNA and protein in human PBL (Figs. 1, 2 and data not shown), consistent with previous findings detecting CXCR4 in a variety of cells including T and B lymphocytes, monocytes, thymocytes, and dendritic cells as well as in human T cell lines such as Jurkat, CEM, and H9 (39–42). Confirming an earlier report by Cole and co-workers (16), we found that DcAMP stimulation of human PBL increased CXCR4 cell surface expression (Fig. 2). In contradiction to this report, however, which failed to find changes in CXCR4 mRNA, we found that cAMP agonists regulated CXCR4 mRNA in an actinomycin D-dependent manner (Fig. 1). Their conclusions (16) were based on RT-PCR data alone, determined at a single time point (20 h) after DcAMP stimulation. In our studies, regulation of CXCR4 mRNA by cAMP agonists was shown to be sensitive to time: maximal stimulation of CXCR4 mRNA was observed 3 h after DcAMP treatment, after which CXCR4 mRNA declined. By 24 h the induction of CXCR4 by DcAMP was not significant (Fig. 1). Even though we used RPAs for many studies, we have confirmed the results shown with RT-PCR (data not shown). We do not think therefore that the differences observed between the published results of Cole et al. (16) and our group are explained by the sensitivity of the methods of detection. We also considered the possibility we were detecting an alternate unspliced variant of the 1.7 kb CXCR4 mRNA transcript termed CXCR4-Lo (43). CXCR4-Lo corresponds to a larger 4.0 kb transcript expressed predominantly in PBL and spleen. Using appropriate primers to distinguish CXCR4 from CXCR4-Lo in RT-PCR, a DcAMP-dependent increase of CXCR4 but not CXCR4-Lo mRNA expression was observed in human PBL (data not shown). Thus, we conclude that CXCR4 mRNA is transiently up-regulated after DcAMP treatment and that this up-regulation translates into sustained increases in intracellular and cell surface CXCR4 expression.

Mutational and deletional analysis of the CXCR4 proximal promoter by transient transfection led to identification of a putative CRE element in the 5′ UTR (+41 to +49 nucleotides) of the CXCR4 promoter. All deletion constructs tested except for pGL-CXCR4 (−42) demonstrated up-regulated promoter activity in response to DcAMP treatment. The fact that pGL-CXCR4 (−42) failed to respond to DcAMP implies that *cis*-acting elements within the sequence spanning −93 to −42 are required for CREB-1-dependent *trans*-activation, as has been shown for nuclear respiratory factor 1 (NRF-1) -mediated *trans*-activation of the promoter (22). We also noted an increase in CXCR4 promoter activity in response to stimulation with PMA and ionomycin in all constructs tested with the exception of

pGL-CXCR4 (−42). The data are consistent with reported findings (22, 37). These latter studies demonstrated that the PMA-dependent up-regulation of CXCR4 promoter activity is mediated by the transcription factor NRF-1 (22, 37). Our additional finding that site-directed mutation of the CRE site within the CXCR4 promoter did not affect PMA + ionomycin-dependent promoter activity (Fig. 6) implies that NRF-1-dependent *trans*-activation of CXCR4 is independent of CREB-1 *trans*-activation.

Whereas mutation of the CXCR4 CRE element attenuated the DcAMP-dependent up-regulation of CXCR4 promoter activity, it did not completely abrogate its effects. Although the mutant CRE sequence matched the wild-type putative CRE sequence minimally (3/9 nucleotides), some similarity to the CRE consensus sequence remained (5/8 nucleotides). Perhaps some residual CREB-1 binding below the limits of detection by EMSA allowed some promoter activity. Alternatively, analysis of the CXCR4 promoter sequence revealed other putative CRE elements in the −92 to +59 nucleotide sequence. Multiple CRE sites may contribute to DcAMP-mediated CXCR4 up-regulation.

Our data correlated CXCR4 expression with HIV (X4) viral fusion and subsequent replication (Figs. 3, 4) as shown by others (44–48). It is still possible that receptors other than CXCR4 may correlate with and be responsible for HIV viral fusion. LFA-1 (CD11a/CD18) and ICAM-1 (CD54) expression on target cells has been reported to promote HIV-1 infection and transmission (49, 50). We detected no change in either LFA-1 or ICAM-1 cell surface expression in response to DcAMP stimulation by immunofluorescence and cytometry (data not shown), although other correlates are possible. Our findings do not preclude the possibility that HIV replication is also directly augmented by cAMP-dependent pathways. Indeed, increasing cAMP concentrations has been shown to augment HIV LTR promoter activity as assessed by transient transfection of human PBL and the human T cell Jurkat line using an HIV LTR promoter fused to a luciferase reporter (refs 51–53 and data not shown). Thus, cAMP signaling pathways appear to regulate HIV replication both indirectly—via CXCR4 cell surface expression, increased fusion, and syncytia formation—and directly via CXCR4-independent up-regulation of HIV LTR promoter activity.

The effects of cAMP modulation extend beyond CXCR4 expression. RPA analysis (Fig. 1) not only revealed cAMP-dependent regulation of CXCR4, but also demonstrated that the mRNA levels of another chemokine receptor, CCR7, were modulated by cAMP-dependent pathways. CCR7 is expressed on naive T and B lymphocytes (54), memory T cells, and maturing dendritic cells (55, 56). Involved in lymphoid tissue migration, the expression of CCR7 on naive T cells and B cells facilitates homing to lymph nodes in response to its natural ligand, SLC (CCL21, 6Ckine, Exodus-2, or TCA) (55). CCR7 expression was increased by DcAMP in an actinomycin D-sensitive manner that was comparable to CXCR4 (Fig. 1*A*), yet basal and induced CCR7 mRNA levels were lower than that of CXCR4 (Fig. 1*A* and data not shown). In contrast to CXCR4 and CCR7, CXCR3 mRNA levels were found to be low in human PBL and were unchanged by DcAMP treatment (Fig. 1*A*). CXCR3 has been reported to be expressed at high levels on T helper cell (Th)0s and Th1s and at low levels on Th2s (54). It has been proposed that chemokine receptors serve as markers of naive and polarized T cell subsets and that their gene expression regulates tissue-specific migration of effector T cells. We suggest that in addition to TcR and the microenvironment, cAMP signaling pathways differentially regulate the repertoire of chemokine receptors expressed on these T lymphocytes (CXCR4 and CCR7; not CXCR3), affecting the migration of effector T cell subsets.

Our study has provided a mechanism underlying the cAMP-dependent up-regulation of cell surface CXCR4 expression and HIV infectivity. We have demonstrated transcriptional induction of CXCR4 mRNA via the cAMP signaling pathway and shown binding of the transcription factor CREB-1 to a putative CRE site located at the +41 to +49 nucleotides of the CXCR4 promoter. Increasing cAMP concentrations results in increased intracellular and cell surface CXCR4 protein expression on human PBL on CD4+ and CD8+ T cell subpopulations, leading to increased HIV viral fusion and viral replication. Continued efforts to understand the signaling pathways that modulate the expression of this HIV coreceptor will allow us to test specific inhibitors that may serve to block HIV infectivity. FJ

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